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Isolation and chemical characterization of glicentin C-terminal hexapeptide in porcine pancreas

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Using a radioimmunoassay specific for porcine glicentin C-terminal hexapeptide, we isolated a peptide from porcine pancreas and characterized it as the C-terminal 64–69 sequence of glicentin: H-Asn-Lys-Asn-Asn-Ile-Ala-OH. The purification steps included gel filtration, ion-exchange chromatography and HPLC. In each step, the recovery of the desired peptide, radioimmunologically estimated from the respective elution profile, was 71.4–91.7%. The final yield of the hexapeptide was 22 μg (4.3%) from 800 g pancreas. The pancreatic content of this peptide was estimated to be approximately equimolar to that of pancreatic glucagon. No hexapeptide-like component was detected in porcine intestinal extracts. The data confirmed that the processing of pancreatic proglucagon liberates the C-terminal hexapeptide of the intramolecular glicentin sequence in a tissue-specific manner during the production of glucagon.

Glicentin *Glicentin (64–69)* *Proglucagon* *Isolation* *Radioimmunoassay* *Sequence analysis* *Proteolytic processing*

1. INTRODUCTION

Glicentin, a 69 residue polypeptide, has been isolated from porcine small intestine as a major component of gut glucagon-like immunoreactants (gut GLIs) [1]. The peptide contains the entire sequence of glucagon in positions 33–61 which is extended at the N-terminus via Lys-Arg with the 1–30 sequence, glucagon-related pancreatic peptide (GRPP), and at the C-terminus via Lys-Arg with a hexapeptide (positions 64–69) [2]. Nucleotide sequence analysis of the cloned cDNA or the gene encoding mammalian pancreatic glucagon revealed the presence of a glicentin sequence in the N-terminal portion of the precursor protein, preproglucagon [3–6]. Isolation of GRPP from porcine pancreas [7] supported the concept of proteolytic cleavage of the glicentin sequence from the precursor molecule to produce glucagon in the pancreas. Here we describe the isolation of a hexapeptide from porcine pancreas and its chemical characterization as the C-terminal 64–69 sequence of glicentin.

2. MATERIALS AND METHODS

High-performance liquid chromatography (HPLC) was performed on a Toyo-Soda SP8700 high-speed liquid chromatograph and the eluates were monitored with a Toyo-Soda UV-8-II spectrophotometer at 210 nm wavelength. Amino acid analysis was carried out with a Hitachi 835 amino acid analyzer using *o*-phthalaldehyde. Peptide sequence analysis was performed on an Applied Biosystems 470A gas-phase protein sequencer connected to a Beckman HPLC instrument. The reagents used included acetonitrile and water for HPLC (Merck, Darmstadt), the reagents for sequence analysis (Applied Biosystems, Foster City, CA) and for peptide synthesis (Protein Research Foundation, Osaka). The synthetic porcine glicentin-related peptides used in this study were

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prepared by conventional solution methodology [8]. Purity of the synthetic peptides was assessed extensively by routine analytical criteria. Details of the synthesis will be described elsewhere.

2.1. *Radioimmunoassay*

Radioimmunoassay specific for glicentin C-terminal hexapeptide was performed as described [9]. In brief: 4-(3-hydroxyphenyl)propionylglicentin(62–69) [HPP-glicentin(62–69)] was radioiodinated by the chloramine T method and purified by gel filtration on Sephadex G-10; antigen-antibody reaction was carried out using anti-glicentin-(49–69) serum R4804, ^{125}I-HPP-glicentin(62–69) as tracer and synthetic glicentin(64–69) as standard; antibody-bound antigen was separated from free antigen by dextran-coated charcoal. Relative crossreactivities of various porcine glicentin-related peptides in the radioimmunoassay are shown in table 1.

Pancreatic glucagon specific radioimmunoassay was carried out as described [10] using antisera OAL123.

Table 1

Relative crossreactivities of synthetic and natural porcine glicentin-related peptides in glicentin C-terminal hexapeptide specific radioimmunoassay

Peptide	Relative crossreactivity
Synthetic peptides	
Glicentin(64–69)	accepted as 100
Glicentin(62–69)	89.3
HPP-glicentin(62–69)	91.5
Glicentin(58–69)	132
Glicentin(55–69)	108
Glicentin(49–69)	86.2
Glicentin(37–69)	98.6
Glicentin(33–69) (oxyntomodulin)	105
Proglucagon fragment(30–37)	3.45
Natural peptides	
Glicentin	120
Glucagon	0

The relative crossreactivity of each peptide was estimated by comparing the amounts of the peptide and standard glicentin(64–69) for 50% inhibition of antibody binding of the tracer

2.2. *Extraction of tissue*

Fresh porcine pancreas (800 g) was immediately frozen and homogenized with a 5-fold excess of 0.1 M acetic acid in an ice bath. The homogenate was heated in a boiling water bath for 5 min. After cooling, acetic acid was added to a final concentration of 0.5 M and the homogenate was centrifuged. The precipitate was washed with a 3-fold excess 0.5 M acetic acid and the supernatant and washing were combined and lyophilized; the resulting powder was triturated with acetone.

2.3. *Amino acid analysis*

Peptide (100 ng) was hydrolyzed in 6 N HCl (0.3 ml) at 110°C for 24 h in an evacuated tube. The solution was evaporated to dryness and the residue submitted to amino acid analysis.

2.4. *Sequence analysis*

Peptide (200 ng) was analyzed according to operation programs 01PREP and RUNTFA provided for the sequencer. PTH-amino acids liberated were identified by HPLC on an Ultrasphere column (Beckman Instruments, Palo Alto, CA) (2 × 250 mm) heated at 50°C with use of a gradient solvent system of acetonitrile/20 mM sodium acetate (pH 4.5) (10:90, v/v, for 0–1 min; 10:90–35:65 for 1–3 min; 35:65–40:60 for 3–18 min; 40:60 for 18–35 min). The eluates were monitored at wavelengths of 260 and 322 nm.

3. RESULTS

Glicentin C-terminal hexapeptide-like immunoreactivity in the crude extract of porcine pancreas is shown in table 2 together with pancreatic glucagon immunoreactivity. Acid-alcohol extraction was more efficient for pancreatic glucagon; hexapeptide immunoreactivity was extracted with dilute acetic acid and compared with glucagon immunoreactivity extracted with acid-alcohol. The molar ratio between both immunoreactivities (underlined in table 2) was 1:1.08.

Fig.1 shows a Sephadex G-50 column elution profile of hexapeptide-like immunoreactivity from the extract. The major immunoreactivity coeluted with synthetic glicentin(64–69) and was estimated to be 83.2% of the total hexapeptide-like immunoreactivity applied. In addition, 2 smaller peaks were detected: a very small one coeluted with

Table 2

Glicentin- and glucagon-related immunoreactivities in crude extracts of porcine pancreas

Radioimmunoassay	Immunoreactivity (fmol eq/mg wet wt tissue)	
	A	B
Glicentin C-terminal hexapeptide specific	483	462
Pancreatic glucagon specific	325	524

A, extract with hot dilute acetic acid; B, extract with acid-alcohol (see [12])

glicentin (fraction 33) and the other eluted between glicentin and glucagon (fraction 42). The latter medium-sized peak is very likely to represent the 37-residue peptide, oxyntomodulin, whose presence in pancreas has been suggested [11]. Peak fractions 48–59 were collected and successively fractionated by gel filtration on a Sephadex G-10 column (fig.2). Combined fractions 25–34 were chromatographed on an SP-Sephadex C25 column (fig.3). The immunoreactive peak fractions (76–89) were desalted with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA). The material was then chromatographed on QAE-Sephadex A-25 column (fig.4). Fractions 13–22 were combined and chromatographed on a Sephadex G-15 column (fig.5). The immunoreactive component eluted in fractions 25–28 was finally purified by HPLC on a Nucleosil $5C_{18}$ column (M. Nagel, Düren, FRG). A single peak was detected between retention time 18 min 25 s and 19 min 15 s by immunoreactivity (fig.6a) which corresponded to the main peak when monitored by absorption at 210 nm (fig.6b). This material was collected and found to coelute with synthetic glicentin(64–69) in HPLC (fig.7a–c).

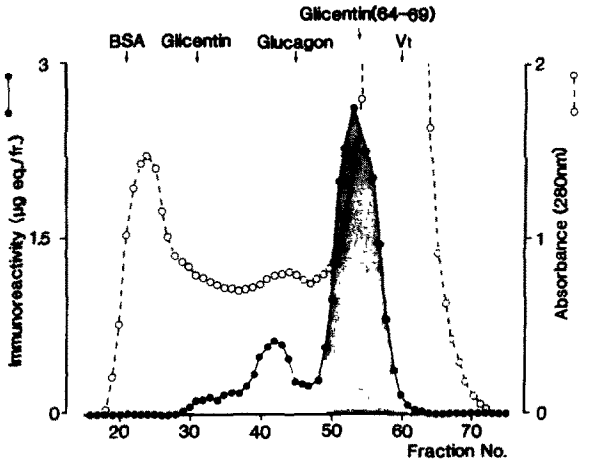

Fig.1. Gel filtration of pancreatic crude extract (1 g) on Sephadex G-50 medium. Column, 3.1 × 110 cm; elution, 3 M AcOH; fraction, 13 g/tube.

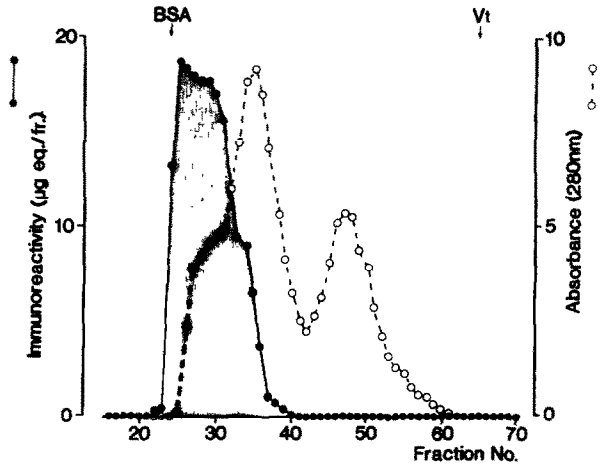

Fig.2. Sephadex G-10 gel filtration of the material (15 g) contained in fractions 48–59 from Sephadex G-50 gel filtration. Column, 3.0 × 93 cm; elution, 3 M AcOH; fraction, 10 g/tube.

The purified peptide preparation was submitted to amino acid and sequence analyses. The results are shown in fig.8.

The data clearly indicate that the isolated peptide was a hexapeptide of the glicentin(64–69) sequence. The yield in each purification step is shown in table 3.

4. DISCUSSION

We have previously developed a radioimmunoassay exclusively specific for the C-terminal hexapeptide portion of glicentin (see table 1) and demonstrated the existence of a glicentin C-terminal hexapeptide-like component in porcine pancreas [9]. Availability of this highly specific radioimmunoassay enabled us to isolate the hexapeptide, glicentin(64–69), from porcine pancreas.

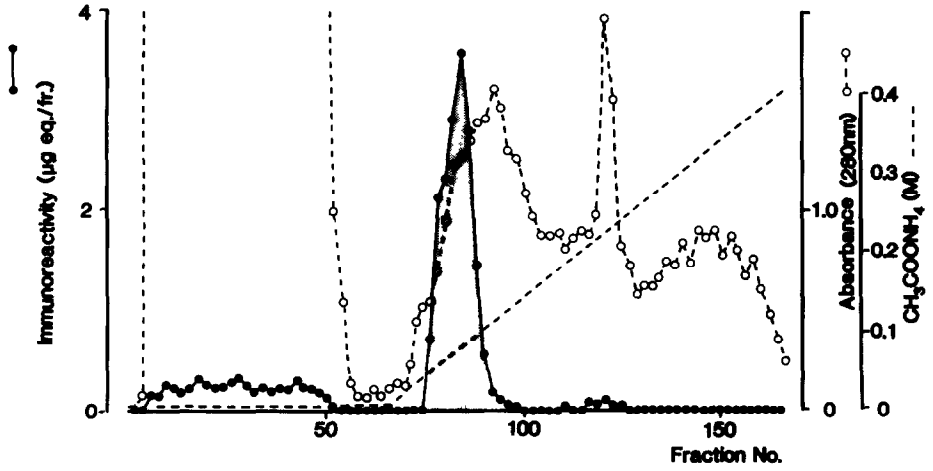

Fig.3. Chromatography on SP-Sephadex C25 of the material (3.57 g) obtained from fractions 25–34 of Sephadex G-10 gel filtration. Column, 3.0 × 23 cm; elution, 0.005 M $AcONH_4$, pH 6.7 (300 ml) followed by a linear gradient with 0.005 M (1000 ml) and 0.4 M (1000 ml) $AcONH_4$, pH 6.7; fraction, 20 g/tube.

The purification steps for the hexapeptide included gel filtration, ion-exchange chromatography and HPLC. During the course of purification, the chromatographic fractions were monitored by radioimmunoassay. In each step, the recovery of the desired peptide, radioimmunologically estimated from the elution profile, was 71.4–91.7%. The final yield of the hexapeptide was 22 μg (4.3%) from 800 g pancreas. The hexapeptide-like immunoreactivity measurable by the present radioimmunoassay in the crude pancreatic extract corresponded to 92.2% (see table 2), on a molar basis, of the pancreatic glucagon immunoreactivity. The immunoreactivity attributable to the hexapeptide itself was estimated to be 83.2% (see table 3) of the total hexapeptide-like immunoreactivity in the crude extract, which in turn corresponded to 76.7%, on a molar basis, of the pancreatic glucagon immunoreactivity. As a result, it seems probable that the hexapeptide and glucagon exist in pancreas on an equimolar basis.

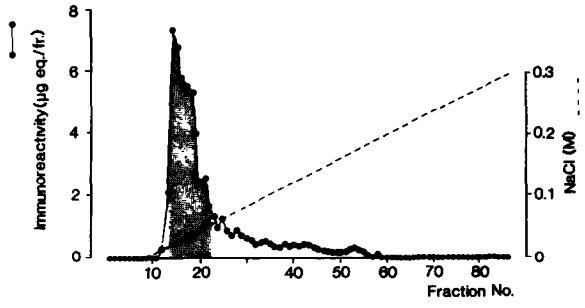

Fig.4. Chromatography on QAE-Sephadex A-25 of the material (15 mg) obtained from fractions 76–89 of SP-Sephadex chromatography. Column, 1.2 × 16 cm; elution, 0.01 M Tris-HCl buffer, pH 9.0 (20 ml), followed by a linear gradient with 0.01 M Tris-HCl buffer, pH 9.0 (175 ml) and the same buffer containing 0.3 M NaCl (175 ml); fraction, 5 g/tube.

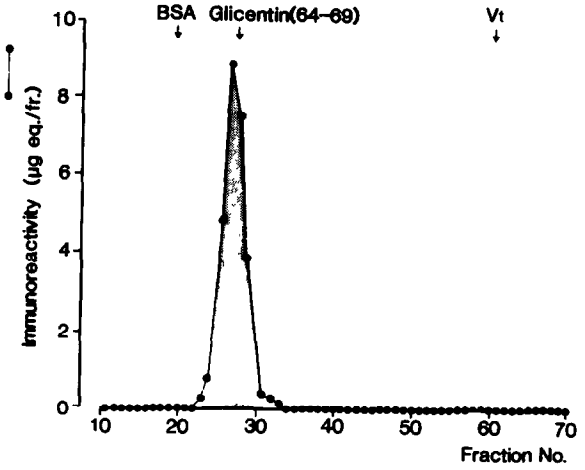

Fig.5. Sephadex G-15 gel filtration of the material obtained from fractions 13–22 of QAE-Sephadex chromatography. Column, 1.9 × 90 cm; elution, 3 M AcOH; fraction,4.5 g/tube.

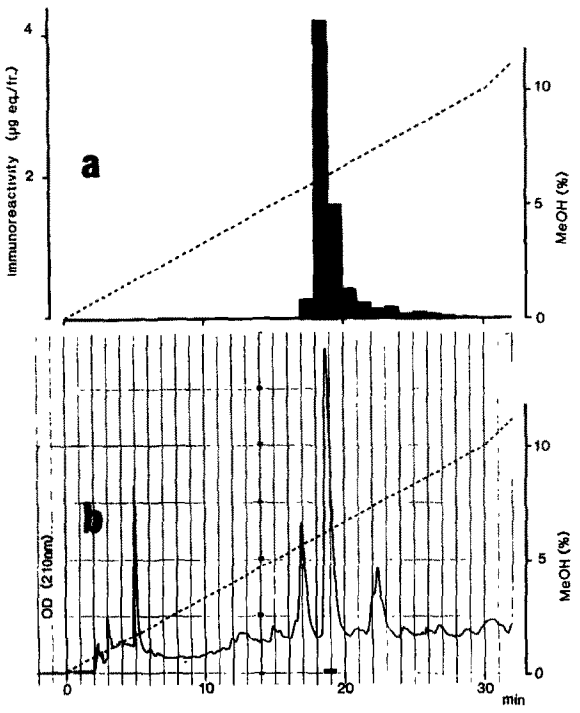

Fig.6. Reverse-phase HPLC of the material obtained from fractions 25–28 of Sephadex G-15 gel filtration. Column, Nucleosil $5C_{18}$ (4.6 × 250 mm); elution, 0.01 N HCl/CH_3OH (100:0–90:10, v/v, for 30 min and 90:10–50:50 for 40 min); flow rate, 1.0 ml/min. The fractions collected every 30 s were measured (a) by radioimmunoassay and the eluate was monitored (b) by absorption at 210 nm.

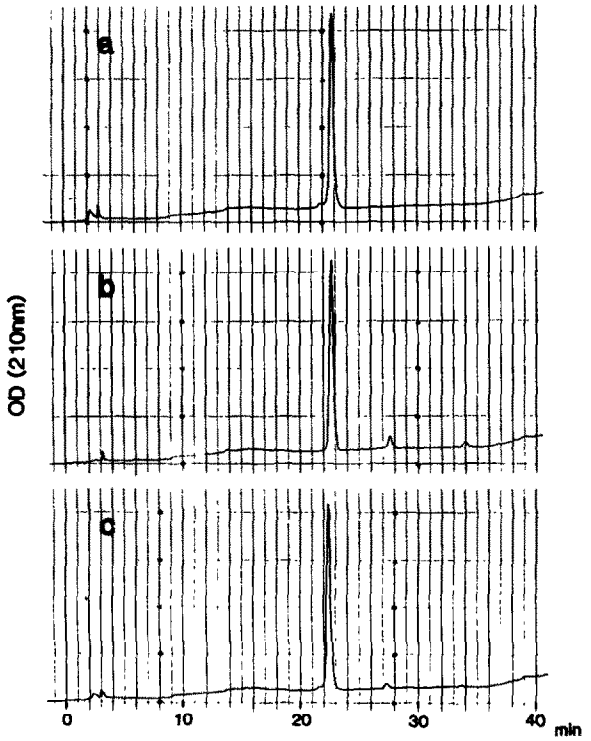

Fig.7. Identification by reverse-phase HPLC of purified peptide with synthetic H-Asn-Lys-Asn-Asn-Ile-Ala-OH. Column, Nucleosil $5C_{18}$ (4.6 × 250 mm); elution, 0.01 N HCl/CH_3OH (100:0–90:10, v/v, for 30 min and 90:10–50:50 for 40 min); flow rate, 1.0 ml/min; detection, 210 nm. (a) Purified peptide (2 μg), (b) synthetic H-Asn-Lys-Asn-Asn-Ile-Ala-OH (2 μg) and (c) purified peptide (1 μg) + synthetic hexapeptide (1 μg).

No hexapeptide-like component was detected in porcine intestinal extracts, which was compatible with a previous suggestion on the formation of oxyntomodulin in porcine intestine [12]. These data indicate that the processing of pancreatic proglucagon liberates the hexapeptide from the glicentin sequence in a tissue-specific manner during the production of glucagon. Moody et al. [13] have suggested that the glicentin N-terminal 30-residue peptide GRPP is a major cleavage product of porcine proglucagon. Our study revealed the C-terminal hexapeptide of glicentin as another major processing product of the precursor. Lopez et al. [3] and Bell et al. [4] have independently proposed the biosynthetic pathway for the processing of mammalian pancreatic preproglucagon. More recently, Patzelt and Schiltz [14] have also described the conversion of proglucagon in rat pancreatic A cells. However, all these investigators have left the fate of the hexapeptide during processing uncertain. The present isolation of the hexapeptide corresponding to glicentin(64–69) con-

Amino acid ratios in acid hydrolysate

$$Asp_{2.88}Ala_{1.08}Ile_{0.96}Lys_{1.09}$$

Sequence analysis

Asn-Lys-Asn-Asn-Ile-Ala

Fig.8. Amino acid sequencing of the purified peptide from porcine pancreas.

Table 3

Purification of glicentin C-terminal hexapeptide from porcine pancreas

Purification step	Material obtained	Immuno-reactivity[a] (μg eq)	Recovery[b] (%)	Yield (%)	Purification (×)
Extraction[c]	37.2 g	490	–	100	–
Sephadex G-50	6.30 g	262	83.2	53.5	3.2
Sephadex G-10	3.57 g	186	73.9	38.0	4.0
SP-Sephadex C25	–	106	71.4	21.6	–
Sep-Pak	15 mg	79.5	77.7	16.2	402
QAE-Sephadex A-25	–	41.6	85.2	8.5	–
Sephadex G-15	–	29.6	73.5	6.0	–
Nucleosil $5C_{18}$	22 μg[d]	21.0	91.1	4.3	72300

[a] Glicentin C-terminal hexapeptide-like immunoreactivity (hexapeptide eq)
[b] Recovery of immunoreactivity in the respective step
[c] From 800 g fresh porcine pancreas
[d] Calculated from absorption at 210 nm in HPLC by comparison with that of synthetic glicentin(64–69)

firmed the intact formation of the peptide with glucagon on proteolytic conversion of proglucagon in the pancreas (fig.9).

We demonstrated [15] by an immunohistochemical protein A-gold technique with the currently used antiserum R4804 that glicentin C-terminal immunoreactivity was located almost exclusively over the peripheral mantle of the α granules of human pancreatic A cells where glicentin N-terminal immunoreactivity was also demonstrated [16]. The present results may suggest that the immunoreactivity observed in the peripheral mantle is attributable to the hexapeptide, not glicentin or oxyntomodulin, formed as a product of proteolytic conversion.

Tager and Steiner [17] have reported the sequence of -----Asn-Asn-Lys-Asn-Ile-Ala-OH for the C-terminal sequence of the proglucagon fragment which they had isolated from porcine/bovine pancreatic glucagon preparation. Together with the putative amino acid sequences of mammalian preproglucagon, the present data also confirmed the Asn-Lys-Asn-Asn-Ile-Ala sequence for the C-terminal extension of glucagon in the precursor molecule in porcine pancreas.

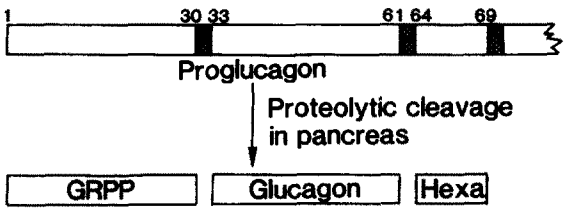

Fig.9. Formation of glicentin C-terminal hexapeptide in pancreas.

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